

*Original Research***Enzyme Immunoassay for Determination of Plasma Total Estrogen in Black Bengal Goat During Peripubertal Period**Avijit Haldar^{1*}, Aditi Patra², Chanchal K. Biswas² and Bukkaaraya S. Prakash³

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Abstract

The current study aims the validation of an enzyme immunoassay (EIA) for the quantification of total estrogen (TE) in blood plasma of Black Bengal goats during peripubertal period. A microtiter plate based competitive assay was developed using a capture EIA for anti-bovine TE and horseradish peroxidase coupled bovine TE. Bovine TE standards were prepared in charcoal-dextran stripped goat plasma. Specificity: A dose-response inhibition curve resulting from serially diluted goat plasma was parallel to the standard curve using bovine TE and thus it confirmed the similar specificity of bovine TE standard and endogenous TE in goat plasma for bovine TE antibody. Sensitivity: The minimum detection level of the assay was 0.10 pg TE/ 50 µl, corresponding to 2 pg/ ml plasma. Precision: The inter- and intra-assay coefficients of variations (for repeatability and reproducibility, respectively) for the pooled plasma samples in 10 assays were 8.5% and 7.3% and 9.7% and 7.3%, respectively. Validation: Pubertal symptoms in goats were coincided with the higher plasma TE level. Plasma TE levels in the goats exposed with buck were higher ($P < 0.05$) than the goats maintained without a buck.

Key words: Black Bengal, Enzyme Immunoassay, Goat, Peripubertal Period, Total Estrogen**How to cite:** Haldar, A., Patra, A., Biswas, C., & Prakash, B. (2020). Enzyme Immunoassay for Determination of Plasma Total Estrogen in Black Bengal Goat during Peripubertal Period. International Journal of Livestock Research, 10(3), 160-175. doi: 10.5455/ijlr.20191125120939**Introduction**

Estrogen hormone plays an important role in animal reproduction. It is produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex, and the male testes. Estradiol is secreted into the

blood stream where 98% of it circulates bound to sex hormone binding globulin (SHBG). To a lesser extent, it is bound to other serum proteins such as albumin. Only a tiny fraction circulates as free hormone or in the conjugated form (Siiteri *et al.*, 1982). Estrogenic activity is affected via estradiol-receptor complexes which trigger the appropriate response at the nuclear level in the target sites. These sites include the follicles, uterus, mammary glands, vagina, urethra, hypothalamus, pituitary and to a lesser extent the liver and skin. This steroid hormone is actively involved in feedback mechanism for influencing the secretion of the gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH), which are essential for follicular maturation and ovulation, respectively, in goats (Medan *et al.*, 2003) and behavioral events associated with estrous cycle (Imwalle and Katz, 2004). Besides, serum estradiol measurements are a valuable index in evaluating a variety of ovarian dysfunctions such as precocious or delayed puberty or anestrus condition (De Santiago-Miramontes *et al.*, 2011; Murtaza *et al.*, 2019).

The levels of total estrogen (TE) in peripheral caprine plasma are very low. Commonly, TE at a very low concentration is estimated in blood plasma by employing radioimmunoassay (RIA) which requires the leveling with the isotope. Besides, a very small quantity of plasma needs to be extracted and processed with complicated methods prior to the assay. Although this method is reliable and accurate, specialized laboratory is needed to use radioisotopes. The availability of radioisotope is also a limitation for TE RIA. Few simple and quick EIA using second-antibody coating with horseradish peroxidase as the label have been validated as having better sensitivity than RIA for the estimation of estradiol- 17β and/ or TE in the plasma of cattle (Meyer *et al.*, 1990), buffalo (Roy and Prakash, 2009) and mithun (Mondal *et al.*, 2006). There is a scanty of information on development of EIA using horseradish peroxidase (HRP) as the marker enzyme for the determination of TE in goat plasma. Therefore, the objective of this study was to develop and validate an EIA for the determination of TE concentration in goat plasma. As sexually active bucks are able to induce estrus in goats (Carrillo *et al.*, 2011; Bedos *et al.*, 2012), the physiological validation of this EIA technique was tested by estimating TE in blood plasma of goats reared with buck and without buck during the peripubertal period.

Material and Methods

Second Antibody

Goat IgG anti-rabbit IgG (second antibody), prepared by Anandlaxmi and Prakash (2001), was obtained from Dr. B.S. Prakash, National Dairy Research Institute, Karnal, Haryana, India.

Hormonal Antibody

The antisera against TE (Es-6-CMO-BSA; E₂/3/pool 1) was generously gifted by Dr. H.H.D. Meyer, Institute Fuer Physiologie, Freising-Weihestephan, Germany for the present study. The cross-reactivities of the anti-Es-6-CMO-BSA with several endogenous steroids are summarized in Table 1.

Table 1: Cross-reactivity of the anti-Es-6-CMO-BSA with several endogenous steroids

Steroid	Cross-reactivity
Oestradiol-17 β	100%
Oestradiol-3-benzoate	50%
Oestradiol -17 α	0.90%
Estrone	0.70%
Estriol	<0.1%
Trembolone	1%
Testosterone	0.50%
Zeranol	<0.25%

Horseradish Peroxidase Total Estrogen Conjugate

Horseradish peroxidase (HRP; Serva, Germany) was used for coupling to TE using the mixed anhydride method (Liebermann *et al.*, 1959), as modified by Meyer *et al.* (1986). Horseradish peroxidase total estrogen conjugate was generously gifted by Dr. H.H.D. Meyer, Institute Fuer Physiologie, Freising-Weihenstephan, Germany.

Total Estrogen Standards

Bovine total estrogen (bTE) standards ranging from 0.10 to 100 pg/well/50 μ l of plasma corresponding to a range between 2 and 2000 pg/ml were prepared in charcoal-dextran stripped goat plasma (Haldar *et al.*, 2009).

Stock Solutions

The buffers and solutions used in the assay are presented in Table 2.

Table 2: Different buffers and solutions used in the enzyme immunoassay

S. No.	Reagent	Formula
1	Coating buffer	15mM Na ₂ CO ₃ , 35mM NaHCO ₃ ; pH 9.6
2	Phosphate buffer saline (PBS)	10mM NaPO ₄ , 0.5M NaCl; pH 7.2
3	Assay buffer	50 mM NaPO ₄ , 0.15M NaCl, 0.02% thiomersal (Sigma-Aldrich, Germany); pH 7.2
4	Blocking solution	1% Bovine serum albumin (BSA) (Merck, Germany) in PBS
5	Substrate buffer	0.05M citric acid, 0.11M Na ₂ HPO ₄ , 0.05% hydrogen peroxide urea (Merck, Germany); pH 4.0
6	Tetra methyl benzidene (TMB) solution	12.5mg 3,3',5,5'-tetra methyl benzidene (Merck, Germany) per ml of dimethyl sulfoxide (Merck, Germany)
7	TMB substrate solution	340 μ l TMB solution in 17 ml substrate buffer
8	Washing solution	0.05% Tween 20 (Sigma-Aldrich, Germany) in distilled water

Preparation of hormone-free goat plasma

To prepare TE free goat plasma for use in assay standard curve, plasma was prepared from blood samples collected in heparinized tubes (20 IU heparin/ml blood) from two aged, non-pregnant, dry, anoestrus goats

(6 kiddings completed), in which the circulating TE concentration was anticipated to be minimal. The plasma was separated by centrifugation of the blood at $2500 \times g$ for 10 min at 4°C . To remove any trace of TE as well as other interfering hormone molecules in the plasma, the plasma was treated with an activated charcoal and dextran mixture (Haldar *et al.*, 2009) and such charcoal-dextran stripped goat plasma was collected and stored at -20°C for future use.

Benzene Extraction of Plasma Samples

The goat plasma samples (1 ml) were taken in duplicate in 15 x 125 mm glass tubes and benzene (6 ml) was then added to each tube containing the plasma sample. The tubes were then vortexed for 2 min and allowed to stay for 1 min. Five milliliters of the upper organic layer were pipetted out in another set of 12 x 100 mm glass tubes. The benzene was then evaporated to dryness in a hot air oven at 50°C . The residue was dissolved in 300 μl EIA assay buffer, and the tubes were vortexed for 30 sec to complete solubilization.

Plasma Interference Test

bTE standards in the assay buffer and in 25 and 50 μl of charcoal-dextran stripped goat plasma were assayed to determine any interference of plasma with the accuracy of the assay.

Test of Parallelism between bTE Standard and Endogenous TE in Goat Plasma

The specificity of bTE standards and endogenous TE in goat plasma for bTE antibody was assessed by determining parallelism between bTE standards and endogenous goat plasma TE. Goat plasma samples containing high concentrations of endogenous TE were collected from three goats at estrus and pooled. The pooled sample was serially diluted with assay buffer to obtain plasma dilutions of 1: 0, 1: 1, 1: 2, 1: 4, 1: 8 and 1: 16 and run in an assay along with the bTE standards ranging from 0.10 to 100 $\text{pg}/50 \mu\text{l}/\text{well}$ (corresponding to 2 to 2000 pg/ml) prepared in the assay buffer.

Precision and Sensitivity of the Assay

The intra- and inter-assay coefficients of variation (for repeatability and reproducibility, respectively) were determined in 10 assays using pooled plasma samples containing two TE concentrations (16 and 2 pg/ml). The sensitivity of the assay was recorded from the lowest value statistically different from the zero, computed from the dose corresponding to B_0 minus $t \times \text{SD}$ of B_0 values.

Immunoassay Procedure

First Coating with Second Antibody

A ninety-six well microtiter plate (Nunc, Genetix Biotech Asia Pvt. Ltd., India) was first coated with goat IgG anti-rabbit IgG at a concentration of $1 \mu\text{g}/100 \mu\text{l}$ of coating buffer/well using a 8- channel micropipette (Eppendorf, Germany). The plate was allowed to stand sealed at 4°C overnight.

Second Coating

The plate was decanted (tapped and blotted) and coated with 300µl of blocking solution for blocking the remaining binding sites and incubated for 30 min at room temperature under continuous shaking using a microtiter plate shaker with incubation facility (Allied Scientific, India).

Washing

The coated plate was decanted and washed twice with 350µl/well of washing solution using an automatic microtiter plate washer (W-2002, Electronic Corporation of India Ltd., India).

Antigen-Antibody Reaction

Duplicate of 50 µl of benzene extracted plasma samples or TE standards ranging from 0.10 to 100 pg/50 µl/well (corresponding to 2 to 2000 pg/ ml) prepared in assay buffer were simultaneously pipetted into respective wells. Thereafter, 100 µl of HRP total estrogen conjugate diluted 1: 4,000 in assay buffer was added into each well. The 100 µl of hormone-specific antiserum diluted in assay buffer (1: 20,000) was added immediately to all wells except the wells marked for non-specific binding (NSB). The plate was then covered with parafilm and aluminum foil and subjected to constant gentle agitation for 30 min under dark condition and then kept overnight for incubation at room temperature.

Washing

In the next day, the plate was decanted and washed four times with washing solution using the microtiter plate washer.

Enzyme-Substrate Reaction

A volume of 150 µl of the tetra methyl benzidine (TMB) substrate solution was added to all the wells and mixed gently for 10 sec. The plate was incubated further in the dark for 40 min at room temperature.

Stopping of Enzyme-Substrate Reaction

The enzyme-substrate reaction was stopped by the addition of 50µl of 4N H₂SO₄ to all the wells and mixed gently for 30 sec. It was important to make sure that all the blue color changed to yellow color completely.

Measurement of Optical Density

The optical density (OD) of the yellow colour obtained due to enzyme-substrate reaction was measured at 450 nm within 10 min with the help of a fully automatic microtiter plate reader (MS 5605A, Electronic Corporation of India Ltd., India).

Physiological Validation of the Assay

To evaluate the suitability of the assay for measuring different levels of TE in plasma, twelve female Black Bengal goats, aged 20 weeks old with a body weight ranging from 4.9 to 5.6 kg, were selected randomly from the experimental farm of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India located at a 23°65'N latitude and 88°22'E longitude. The selected animals were checked clinically and confirmed that they were free from any anatomical, physiological or infectious disorders. The goats were divided into two groups having six goats in each group. The goats were fed according to the recommendations of NRC (2007) with an access to grazing on natural pasture and supplementary concentrate feeding having 21% crude protein @ 10g per kg body weight per day. Fresh drinking water was available *ad libitum* to all the goats. The first group of goats was kept with a buck to induce 'buck effect' (Carrillo *et al.*, 2011; Bedos *et al.*, 2012). The other group of goats was not allowed to have an access with a buck. Some estrus behavioral signs were recorded for initial identification of the goats that were in puberty onset (Haldar *et al.*, 2014). The goats that exhibited puberty onset were subjected to transrectal ultrasonographic (USG) examination at 10 days after observed puberty, using 7.5 MHz linear transducer with B-mode to observe the presence of corpus lutea (CL) on ovaries (Orita *et al.*, 2000). The blood samples were collected from all the experimental goats between 09:30 and 10.30h by jugular venepuncture into heparinised polypropylene tubes (20 IU heparin/ml of blood) initially at biweekly intervals starting from 20 weeks of age till 38 weeks of age and then at weekly intervals up to 55 weeks of age to monitor various hormonal status of the animals during the peripubertal period. Following blood collection, the tubes were put on ice and transferred to the laboratory. Blood samples were centrifuged at 2500 x g for 10 min at 4°C. Plasma was separated and stored at -20°C until hormone assay. The experimental protocol and animal care were met in accordance with the National guidelines for care and use of Agricultural Animals in Agricultural Research and Teaching as approved by the Ethical Committee for Animal Experiments (ECAE).

Plasma LH Estimation

Plasma LH concentrations were estimated using a double antibody EIA, using ovine LH for biotinylation and as a standard reference, as previously developed in this laboratory (Haldar *et al.*, 2009). Eighty microliter of plasma sample in duplicate was run in 96-well microtiter plate for the assay. The intra- and inter-assay coefficients of variation (CVs) were 7.5% and 10.3%, respectively. The sensitivity of the EIA assay for LH was 0.3 ng/ml.

Plasma FSH Estimation

Plasma FSH concentrations were quantified by an EIA using commercially available kits for ovine (M/s. Endocrine Technologies, Inc., Newark, CA, USA), as previously validated in this laboratory (Haldar *et al.*,

2013). Fifty microliter of plasma sample in duplicate was used to quantify plasma FSH concentrations by an EIA. The sensitivity of the assay for FSH was 0.5 ng/ml. The intra- and inter-assay coefficients of variation (CVs) were 8.3% and 11.4%, respectively.

Statistical Analyses

Standards and samples were run in duplicate, and mean values were used. Concentrations of hormone were calculated by log/logit transformation of the standard curve, according to the equation, $\text{logit}(Y) = \text{LOG}(p/(1-p)) = \text{Intercept} + BX$, where $Y = \% \text{ binding of bTE}$, $p = \text{the probability of 1}$, $B = \text{the slope}$, $X = \text{the natural log of concentration}$. Parallelism was tested by a chi square test and Pearson goodness-of-fit chi square was applied while fitting logit model. The plasma hormone data from the experimental goats were analyzed considering time as the main effect by an ANOVA for repeated measures technique with post hoc analysis using SPSS 10.0 Statistical Software Package, 1997, SPSS, Inc., USA.

Results and Discussion

The present report describes immunological and physiological validation of an EIA technique for the estimation of goat plasma TE levels. The heterogeneity of molecular isomers of circulating TE, the epitope specificity of the antibodies and the interference from matrix components such as TE binding molecule in plasma are among the reasons why standardizing TE immunoassay is difficult. The present EIA technique uses HRP as the marker enzyme for the determination of TE in goat plasma.

A Standard Curve

A representative log transformed bTE standard curve with a range of 0 and 2000 pg/ml is set out in Fig. 1. The optical density (OD450) of the 0 point was recorded as 1.43. The OD450 for the non-specific binding using 50 μl plasma was low, ranging from 0.19 to 0.22.

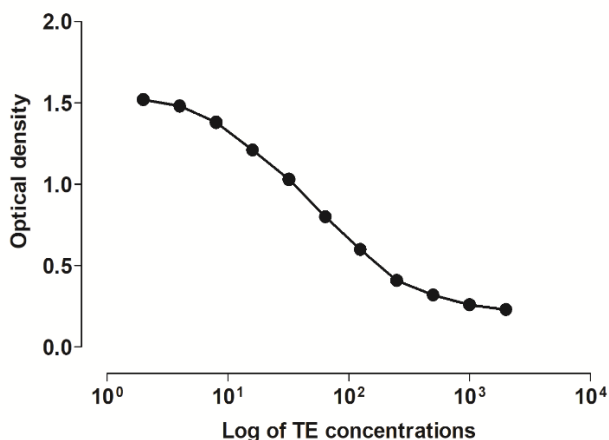


Fig. 1 A representative log transformed standard curve for the enzyme immunoassay to determine TE in goat plasma. Optical density was measured at 450 nm.

The concentration of antibody and antigen are the most crucial for any enzyme immunoassay. As the antigen- antibody binding capacity as well as enzyme activity depended on temperature, pH and even the type of antigen and antibody used in the assay, the present titer level of bTE antibody and HRP- bTE conjugate might differ from the titer level as reported earlier (Mondal *et al.*, 2006; Roy and Prakash, 2009). In the present assay procedure, repeated two-dimensional titer tests indicated that the bTE antibody titer of 1: 20,000 and HRP- bTE conjugate titer of 1:400 were the most suitable.

Test for Plasma Interference with the Assay

Bovine TE standards in the assay buffer and in two volumes of hormone-free goat plasma (25 and 50 μ l) were run in the assay to determine the possible interference of plasma with the accuracy of the assay. As shown in Fig. 2, the plasma interference test while fitting logit model for three standard curves revealed that there was no significant difference ($P= 0.47$) in slopes for standard curves. The percentage binding of different bTE standards in 50 μ l goat plasma was similar to that observed in 50 μ l assay buffer (Fig. 3). The 50% binding point of the standard curves prepared in buffer and undiluted plasma was at 125 pg/ml bTE. Smaller plasma volume (25 μ l) showed slight variation in the percentage binding from the buffer curve. Thus, there was a slight decrease in the accuracy when measuring bTE standards prepared in 25 μ l plasma volumes. The background caused by non-specific binding was low for undiluted and diluted plasma (OD450 ranging from 0.19 to 0.22). Hence, TE standards ranging from 0.10 to 100 pg/well/50 μ l were prepared in hormone-free plasma and subsequently all assays were conducted, using 50 μ l of the unknown plasma samples.

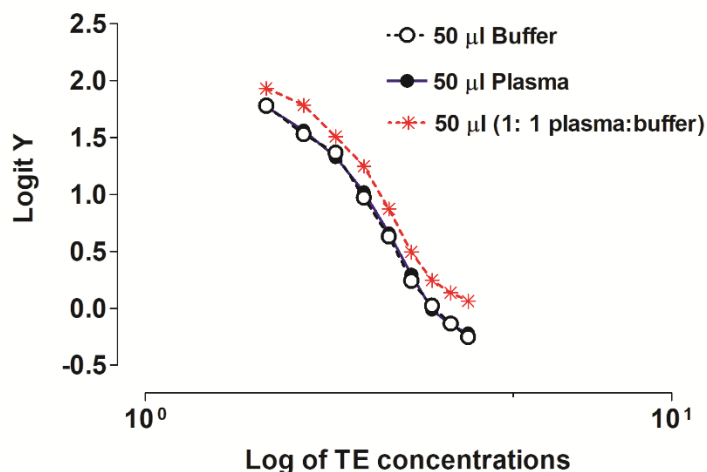


Fig. 2: Log transformed responses of different volumes of plasma on TE standard curves prepared in 50 μ l buffer (O), 50 μ l undiluted plasma (●) and 50 μ l 1: 1 plasma: buffer (*). Optical density was measured at 450 nm.

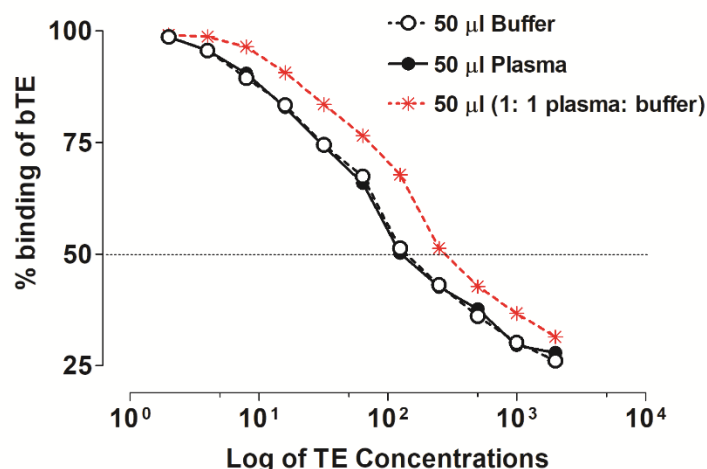


Fig. 3: Influence of different TE concentrations prepared in 50 µl buffer (○), 50 µl undiluted plasma (●) and 50 µl 1: 1 plasma: buffer (★) on the percentage binding in the TE standard curve showed that the percentage binding of different concentrations of bTE in 50 µl undiluted plasma (●) was similar to that observed in 50 µl buffer standards (○). Optical density was measured at 450 nm.

The plasma interference test while fitting logit model for three standard curves (Fig. 2) revealed that there was no difference ($P > 0.05$) in slopes for standard curves obtained from assay buffer, undiluted plasma and diluted plasma indicating no interference of plasma, irrespective of plasma volume used, on the standard curves. The percentage binding of different bTE standards in 50 µl goat plasma was similar to that observed in 50 µl assay buffer (Fig. 3). However, smaller plasma volume (25 µl) showed slight variation in the percentage binding from the buffer curve and there was a slight decrease in the accuracy when measuring bTE standards prepared in 25 µl plasma volume. The effective concentration at 50% binding of the bTE indicated that there was homogeneity between two standard curves prepared in buffer and undiluted plasma. Since the 50% binding points of the standard curves prepared in 50 µl buffer or undiluted plasma were obtained at lower concentration, all assays were run with 50 µl standard or unknown plasma sample. The similarity in percentage binding of different concentrations of bTE between two standard curves prepared in 50 µl buffer and 50 µl plasma agreed well with the earlier report (Roy and Prakash, 2009).

Parallelism between bTE Standard and Endogenous TE in Goat Plasma

The homology between bTE standards and endogenous TE in goat plasma was assessed by conducting parallelism test. The optical density obtained from increasing dilutions of goat plasma samples was parallel to that obtained with the bTE standards as shown in Fig. 4.

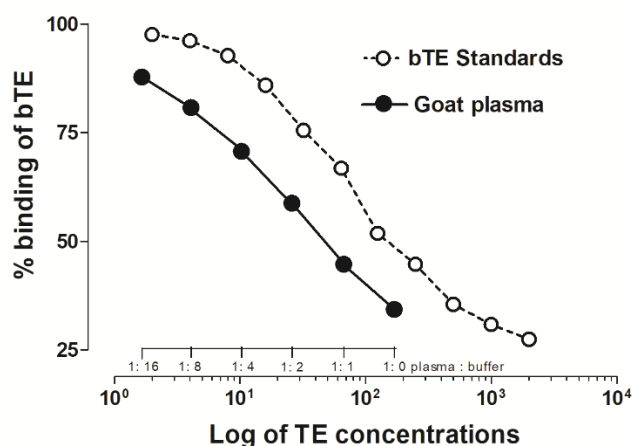


Fig. 4: The curve (●) obtained from the pooled goat plasma sample serially diluted in buffer (1: 0, 1: 1, 1: 2, 1: 4, 1: 8 and 1: 16 plasma: buffer) was parallel to the curve (○) obtained with the bTE standards (ranging from 2 to 2000 pg/ml) in buffer. Optical density was measured at 450 nm.

The parallelism test confirmed a considerable homology between goat and bovine TE used in the assay. Dilutions of goat plasma paralleled bovine TE standards in buffer (Fig. 4) indicating that goat TE was homologous to bTE. The good parallelism obtained with bTE standards confirmed a high degree of homology in the structures of bovine and caprine TE and the assays provided a true and accurate measure of plasma TE in spiked samples of goats (Fig. 5). Hence, the assay provided an actual TE determination in goat plasma. To obtain a high degree of sensitivity of the assay, 50 µl sample volume was ideal in reducing the non-specific binding (OD₄₅₀ ranging from 0.19 to 0.22). This volume was optimum to determine the low physiological baselines of TE concentrations as recorded in goats kept with buck and goats kept without buck (Fig. 5).

Precision and Sensitivity of the Assay

The precision of the assay was evaluated in term of two parameters, namely repeatability (intra-assay coefficient of variation) and reproducibility (inter-assay coefficient of variation). The Inter and intra-assay coefficient of variation determined using pooled plasma in 10 assays were 8.5% and 7.3%; 9.7% and 7.3% respectively. The lowest value statistically different from the zero was recorded at the concentration of 0.10 pg/50 µl/well, which corresponded to 2 pg/ml plasma. The lowest value statistically different from the zero was recorded at the concentration of 0.10 pg/50 µl/well, which corresponded to 2 pg/ml plasma. The intra- and inter-assay coefficients of variation for repeatability and reproducibility, respectively obtained in the present study were comparable to those obtained in enzyme immunoassay to determine plasma TE concentrations in other species (Mondal *et al.*, 2006; Roy and Prakash, 2009).

Physiological Validation of the Assay

The present assay technique was employed to determine TE concentrations in plasma samples of peripubertal goats kept with buck and goats kept without buck. Plasma TE levels (pg/ml) in goats kept with buck and goats kept without buck are presented in Fig. 5. Plasma TE levels of both the groups were fluctuating during the peripubertal period. Plasma TE levels in the goats exposed with buck were significantly higher ($P < 0.05$) than the goats maintained without a buck during 30 to 32 weeks of age. The pubertal symptoms in goats kept with buck were coincided with the higher plasma TE level during 30 to 32 weeks of age. The goats reared without buck showed puberty onset later than the earlier group at about 48 weeks and the pubertal symptoms of goats reared without buck were marked by the high plasma TE level.

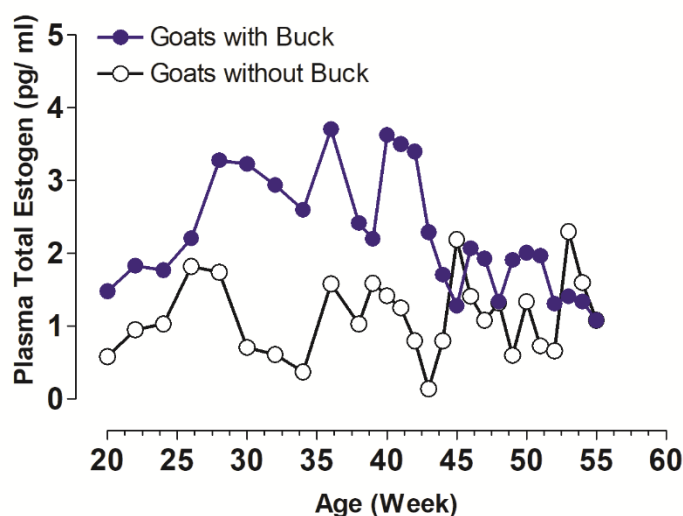


Fig. 5: Average plasma total estrogen level (pg/ml) in goats kept with buck (●) and goats kept without buck (○) from 20 weeks of age to 55 weeks of age. Plasma TE levels in goats exposed with buck were higher ($P < 0.05$) than the goats maintained without a buck during the peripubertal period.

Puberty is the end point of a series of events affecting the development of the ‘hypothalamo-pituitary-gonadal’ axis leading to reproductive competence, which impacts on subsequent reproductive life and productivity of small ruminants (Valasi *et al.*, 2012). Estrogen induces a preovulatory luteinizing hormone surge as an “all or none” event leading to ovulation. Following ovulation, estradiol levels fall rapidly until the luteal cells become active resulting in a secondary gentle rise and plateau of estradiol in the luteal phase (de Castro *et al.*, 1999). With the initiation of cyclicity, estradiol attained higher levels (7.7 ± 1.7 pg/ml) at estrus phase and dropped down to the lower levels and remained below 3 pg/ml in the luteal phase in dwarf goats (Khanum *et al.*, 2008). Oestrus behaviour was positively correlated with the peak concentration of plasma TE during the peri-oestrus period in buffalo heifers (Roy and Prakash, 2009) and in mithun cows

(Mondal *et al.*, 2006). The plasma total estrogen profiles during estrous cycle in goats support the previous report (Bauernfeind and Holtz, 1991). Maternal serum estradiol levels decline to basal levels during the first 4 weeks post-breeding and then increases considerably, with the maximal concentration during late pregnancy (Kandiel *et al.*, 2010).

Plasma FSH concentrations (ng/ml) in goats kept with buck and goats kept without buck are set out in Fig. 6. There was significant difference ($P < 0.05$) in plasma LH concentrations between two groups of goats. Plasma FSH concentrations, in both the groups up to 24 weeks of age, showed almost similar pattern with not much difference. Plasma FSH concentrations in goats kept with buck started to increase from 26 weeks of age with a similar pattern of increasing trend at certain interval during the peripubertal period. The goats kept without buck showed high FSH concentration at 38 weeks of age followed by similar pattern of increasing trend at certain interval over the peripubertal period.

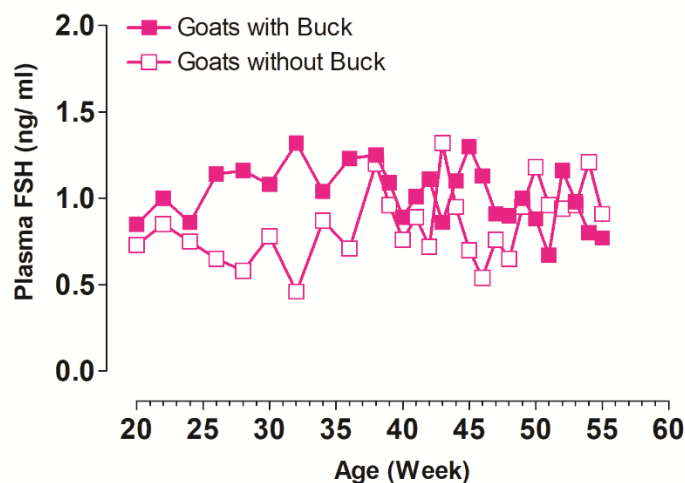


Fig. 6: Average plasma FSH concentrations (ng/ml) in goats kept with buck (■) and goats kept without buck (□) from 20 weeks of age to 55 weeks of age. Plasma FSH concentrations in goats exposed with buck were higher ($P < 0.05$) than the goats maintained without a buck over the time.

Plasma LH concentrations (ng/ml) in goats kept with buck and goats kept without buck are presented in Fig. 7. There was no significant difference ($P > 0.05$) in plasma LH concentrations between two groups of goats. However, a similar pattern of plasma LH surge at certain interval was recorded in both the groups of goats.

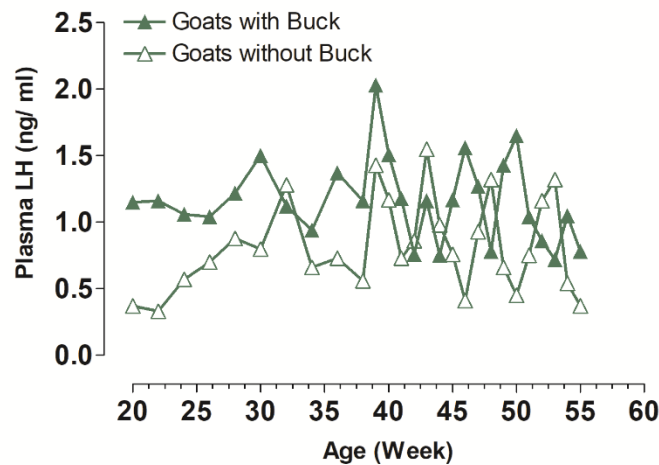


Fig. 7: Average plasma LH concentrations (ng/ml) in goats kept with buck (▲) and goats kept without buck (△) from 20 weeks of age to 55 weeks of age. There was no significant variation ($P>0.05$) in plasma LH concentrations in goats exposed with buck and the goats maintained without a buck over the time.

A representative plasma TE levels (pg/ ml), plasma FSH level (ng/ ml) and plasma LH level (ng/ ml) of a single goat reared with buck is presented in Fig. 8 to identify the pattern of changes in different plasma hormones during peripubertal period.

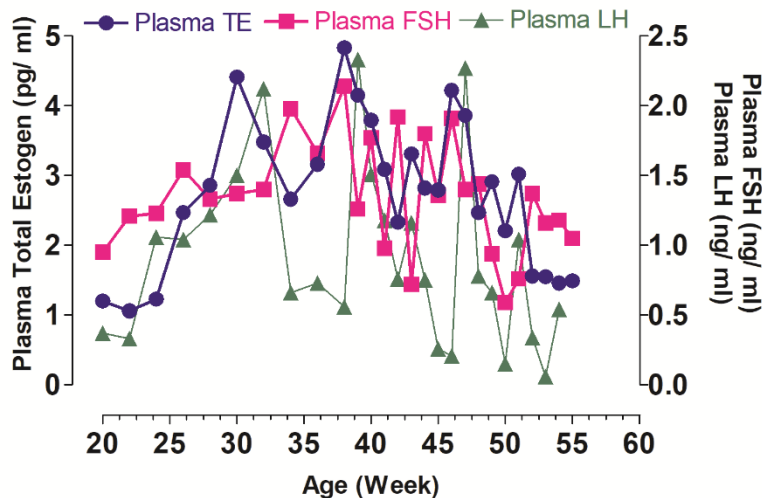


Fig. 8: Plasma total estrogen level (●), plasma FSH level (■) and plasma LH (▲) of a representative goat reared with buck from 20 weeks of age to 55 weeks of age during the peripubertal period.

Interestingly, increased levels of plasma TE, FSH and LH were recorded at certain interval in such goat kept with buck. At 32 week of age, plasma TE, FSH and LH levels coincided with the onset of puberty and subsequent estrous cycles which were confirmed by transrectal ultrasonographic (USG) examination at 10 days after observed estrus. Plasma TE was found to increase from 6 days before estrus to reach a peak level

on the day of estrus and decline thereafter to basal level on day 2 of the cycle. After service, a successful pregnancy was set at 46 week of age, which was also evident from plasma TE level. During the pregnancy period, plasma TE level gradually declined.

In the present study, plasma FSH and LH profiles in peripubertal goats (Fig. 6- 8) agree well with the earlier report (Gaafar *et al.*, 2005; Haldar *et al.*, 2014). Sexually active bucks have been found to induce estrus in goats (Chemineau *et al.*, 2006; Guillen-Muñoz *et al.*, 2018). Daily contact with bucks has also been documented to induce estrus in anoestrus goats (Bedos *et al.*, 2010; Luna-Orozco *et al.*, 2012). Plasma TE profiles in goats during peripubertal period confirmed the biological validation of the present EIA technique for the determination of TE in goat plasma.

Conclusion

A simple and highly sensitive and competitive EIA technique has been developed and validated on second antibody coated 96-well microtiter plate using horseradish peroxidase as the marker enzyme for determination of plasma TE in goat. This EIA technique is convenient, safe, quick (requires only 48 hours) developed for caprine TE. It is a reliable tool for the purpose. This method is especially important in the context of endocrine research in laboratories located in remote areas where access to the radiochemicals, normally required for the conventional RIA procedure, is difficult. Thus, the present EIA technique could be used to determine circulating caprine TE concentrations on routine basis.

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